Exhibit A

ELSEVIER

Does the Presence of Unwanted Dermal Fibroblasts Limit the Usefullness of Autologous Epidermal Keratinocyte Grafts?

T. Drewa, K. Szmytkowska, Z. Wlodarczyk, I. Sir, and C. Kierzenkowska-Mila

ABSTRACT

Objectives. Fibroblasts sometimes occur after enzymatic isolation of epidermis. They proliferate quickly, overgrowing the culture. A pure epithelial culture is essential for therapy using a keratinocyte graft. The aim of this study was to determine the possibility of fibroblast elimination from culture to prevent fibroblast overgrowth and obtain a pure monolayer of keratinocytes.

Material and Methods. We analyzed three epidermal-derived cultures. Cells were cultured in medium contained Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 at a 3:1 ratio with 5% autologous serum and additives. The epithelial culture was confirmed using pancytokeratin MMF. If fibroblast like cells were present, they were removed using 0.01% edetate disodium dihydrate (Na_2EDDA). This procedure was repeated until we obtained pure primary keratinocyte cultures.

Results. Fibroblast detachment was observed after Na_2EDDA treatment. The procedure was performed twice and pure primary cultures of keratinocyte were achieved in two cases. These two cultures maintained their epithelial-like morphology and cytokeratin expression. One culture was treated four times with Na_2EDDA with no effect; the morphology of the cultures became fibroblast-like with no observed cytokeratin expression.

Conclusions. Unwanted dermal fibroblasts can be separated from primary keratinocyte cultures during the first few days after the isolation. Cocultures of unwanted dermal fibroblasts and epidermal keratinocytes can be reverted to pure keratinocyte monolayers suitable as grafts for transplantation.

GRAFTING OF autologous cultured human keratinocytes is a standard treatment for burn patients.[1] Keratinocyte culture is usually performed in a serum-free system, but media supplemented with autologous serum seems to be the safest condition.[2–4] Keratinocytes promote more proliferation and less apoptosis of cocultured fibroblasts through paracrine effects.[5] Up to 4% of human fibroblasts have been observed after enzymatic isolation of a dermis-containing biopsy using a trypsin/EDTA bath, which is the preferred method to isolate keratinocytes from human skin.[6] It was shown that lysates of cultured keratinocytes display mitogenic activity for keratinocytes and fibroblasts.[7] Those fibroblasts usually have a high proliferation rate, sometimes tending to overgrow the culture. Autologous, serum-conditioned media promote fibroblast proliferation. A pure epithelial culture is essential for keratinocyte graft therapy. The aim of this study was to determine the possibility to remove fibroblasts from primary keratinocyte cell cultures to prevent fibroblastic overgrowth and obtain a pure keratinocyte monolayer.

MATERIALS AND METHODS

An analysis was performed on three keratinocyte cultures. Primary human keratinocyte cultures were obtained from three patients and 25 mL blood was collected from each patient. Specimens of approximately 1 cm^2 were harvested during surgical procedures involving excess skin removal (e.g., circumcision). The donor site

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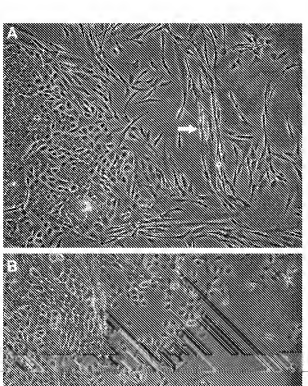

Fig 1. **A.** The keratinocytes and unwanted fibroblasts *(arrow)* in primary coculture 8 days after isolation procedure (inverted microscope, original magnification ×100). **B.** The fibroblasts were removed using gently detachment with 0.01% Na_2EDA. The keratinocytes started to build confluent layer. Day 9th of culture (inverted microscope, original magnification ×100).

was disinfected preoperatively with a 0.5% solution of chlorhexidine in 70% alcohol. Tissue was placed in tubes containing phosphate buffered saline solution (PBS, Sigma, Poznan, Poland). The specimens were incubated overnight in a 0.25% trypsin solution at 4°C. After incubation, the basal layer of the epidermis was scraped off. The scraped off cells were placed in tubes and centrifuged at 500 *g* for 5 minutes. The supernate was removed and the residue suspended in 5 mL of medium containing Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 at a 3:1 ratio, supplemented with 5% autologous serum. The medium was supplemented with insulin, transferin, triiodothyronine, hydrocortisone, EGF, penicillin, and streptomycin (Sigma). The epithelial character of the culture was confirmed by assessment of cytokeratin expression using anti cytokeratin antibodies (Pancytokeratin MMF; Dako, Glostrup, Denmark). The cultures were observed daily under an inverted microscope (Nikon Eclipse TS100; Tokyo, Japan) equipped with a digital camera (Nikon E5400). Each flask was observed carefully to detect fibroblasts during the primary culture period of 3 weeks. In the case of fibroblast-like cells present a procedure of elimination was introduced: namely, fibroblasts were removed using 0.01% (edetate disodium dihydrate (Na_2EDA) Sigma), which was gently dropped onto the fibroblast sheets. The removal of fibroblast-like cells was performed under an inverted microscope until all fibroblast-like cells were detached from the culture. The epithelial-like sheets became intact during the procedure. Then the Na_2EDA was removed and medium added. This procedure was repeated until we obtained pure keratinocyte primary cultures.

RESULTS

Three cocultures with keratinocyte to fibroblast proportions from 10:1 to 8:1 were treated with Na_2EDA (Fig 1A and 2A). In the pretreatment stage, cells were washed using low Ca^{2+} and Mg^{2+} PBS. During this procedure keratinocytes and fibroblasts were still undetached. Fibroblast detachment was observed when the Na_2EDA was gently deposited on the culture surface. The process was performed until all visible fibroblast-like cells were eliminated (Fig 1B and 2B). In two cultures, this procedure occurred twice; finally

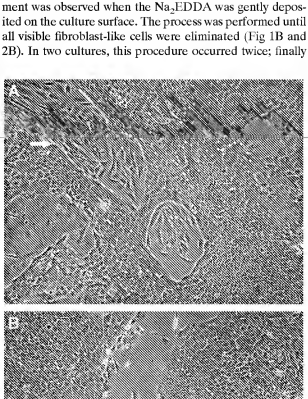

Fig 2. **A.** The islets of fibroblasts are still visible *(arrow)* after the first procedure of fibroblasts removal. Day 15th of culture (inverted microscope, original magnification ×100). **B.** The second procedure of fibroblast removal was successful. The keratinocyte sheets remained intact. Pure keratinocyte culture was obtained (inverted microscope, original magnification ×100).

we achieved pure keratinocyte primary cultures. These two cultures maintained their epithelial-like morphology and cytokeratin expression at the end of the observation period. One culture was treated with Na_2EDDA four times with no effect. In this case the morphology of the entire culture became fibroblast-like. No cytokeratin expression was observed in this fibroblast-like culture.

DISCUSSION

Skin replacement has been a challenging task for surgeons. Pure keratinocyte cultures are necessary for tissue-engineered skin replacements like cultured autologous, allogeneic keratinocyte grafts or autologous/allogeneic composites combined with sealants or scaffolds.[8] The use of cultivated keratinocytes in partial-thickness scald burns minimizes the areas of autologous skin harvesting and reduces the amount of blood transfusion. The application of cultured epithelial autografts to burn wounds is known to reduce scarring and contraction.[9,10] There are several causes of primary epithelial cell culture failure; one is the presence of unwanted fibroblasts, which may overgrow the culture. The probability of fibroblast proliferation in the culture may be increased when autologous serum is used as the supplement. Autologous serum is often used as a safe medium supplement.[2–4] It should be mentioned that a serum-free system is not absolutely safe, because of bovine pituary extract supplementation or different "xenogenic feeder layers" prepared previously on bovine serum.[11]

Fibroblasts are often scraped off together with keratinocytes during the isolation procedure. The amount of unwanted fibroblasts also depends on the enzymatic bath conditions.[6] In this study, we presented a simple method to eliminate unwanted fibroblasts from keratinocyte cultures supplemented with autologous serum. The possibility to detach only fibroblasts was based on the differences in cell–cell adhesions between keratinocytes and fibroblasts in cultures. There are many differences in actin filaments and adherens junctions between fibroblasts and epithelial cells, which are connected with anchorage properties of cells in culture.[12] Epithelial cells are characterized by stronger attachment properties than fibroblasts.[13] Keratinocytes form strong cell–cell adhesions in a calcium-rich environment.[14,15] The formation of adherens junctions and desmosomes requires extracellular calcium. The Na_2EDDA binds Ca^{2+} ions. This study demonstrated that low concentrations of Na_2EDDA can be used to remove unwanted fibroblasts from a coculture: two of three cultures were successfully cleaned of fibroblasts to pure keratinocyte sheets. This method seemed to be suitable for preparation of pure keratinocytes grafts for future transplantation. The separation procedure should be performed when the fibroblast amount is low in culture. It has to be proceeded within the first few days after establishment of the primary culture. Keratinocyte-secreted soluble growth factors dramatically influenced dermal fibroblasts.[16] When the amount of fibroblasts was too high, the cell separation procedure failed. The fibroblast overgrowth was clearly seen in the case of the third culture in this study. The keratinocytes disappeared in the presence of autologous serum with huge amounts of fibroblasts after the second week of culture.

It has been proven by clinical observation that unhealed burn wounds, which lack the epidermis, demonstrate excess collagen production and scarring. Clinical strategies to decrease the hypertrophic scar include an attempt for early application of cultured epithelial autografts. The wound healing processes can be stimulated distinctly by growth factors secreted from keratinocytes, with a prominent role for keratinocyte growth factor.[17,18] The establishment of pure keratinocyte cultures is the crucial element in this strategy.

In conclusion, unwanted dermal fibroblasts may be separated from primary keratinocyte cultures during the first few days after the isolation procedure. Cocultures of unwanted dermal fibroblasts and epidermal keratinocytes can be reverted to pure keratinocyte monolayers suitable as grafts for transplantation.

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